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THE EFFECT OF THE MEDIUM ON APPARENT VITAMIN-SYNTHESIZING DEFICIENCIES OF MICROORGANISMS[1]

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Beadle and Tatum's work ('45) on biochemical mutants of *Neurospora* has resulted in wide acceptance of the view that it is relatively easy to distinguish strains genetically capable from those genetically incapable of synthesizing vitamins. They discovered many mutants apparently incapable of performing specific syntheses. The present paper reveals, however, that different synthetic media so affect the growth of organisms that conclusive demonstration of specific deficiency requires a much more critical study of the environment than has hitherto been achieved. Differential growth of cultures in a synthetic medium which deviates in many respects from the natural substrate may prove very useful for genetical diagnosis but may not give reliable information concerning the synthetic activity of the organism under normal conditions. Without critical testing it is difficult to say whether a given deficiency is absolute. The following data will show that the standard test of ability to grow in the presence and inability to grow in the absence of a given vitamin in a synthetic medium is inadequate and that the criterion of equal increments of growth for equal additions of the vitamin may give an ambiguous answer.

A COMPARISON OF TWO SYNTHETIC MEDIA

Table I shows the formulas of the synthetic media used by Burkholder ('43), Hutner (unpublished), Wickerham ('46), and Beadle and Tatum ('45). Many experiments have shown that Hutner's medium is a very different substrate for yeast growth from Burkholder's. This was clearly revealed by growing cultures Nos. 3 and 10 (Table II) on two batches of each medium, one to which no pantothenate was added and the other containing 50 γ of pantothenate per liter (fig. 1). Culture No. 3 does not begin to grow in Burkholder's medium without

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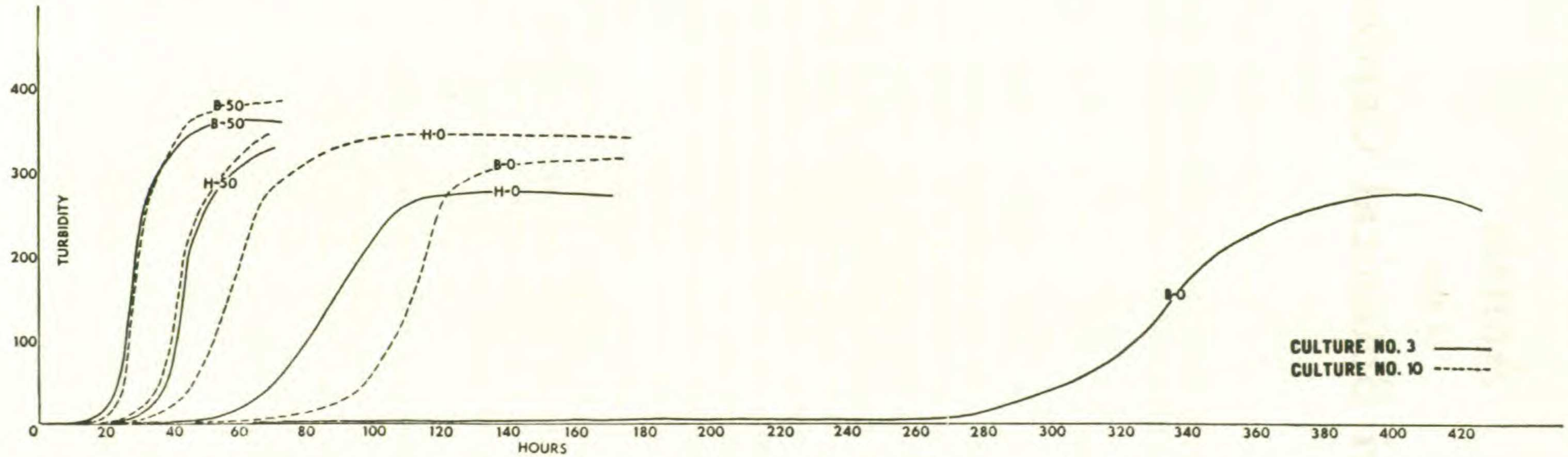

Fig. 1. Comparison of the amounts of growth on Burkholder's and Hutner's media. The turbidity indicated by a Klett Photoelectric Colorimeter is plotted against the time in hours after inoculation. Culture No. 3 was grown on two batches of Hutner's medium, one containing 50 γ of pantothenate per liter and the other containing no added pantothenate. It was also grown on two different batches of Burkholder's medium, one containing 50 γ per liter of pantothenate and one no added pantothenate. Culture No. 10 was also grown on these four different kinds of media.

added pantothenate until after 200 hours, but growth is completed in Burkholder's medium containing 50γ of pantothenate per liter after 50 hours. Readings taken at 72 hours would be interpreted to mean that it was a "nonsynthesizer." Culture No. 10 is capable of more rapid growth than culture No. 3 in Burkholder's without added pantothenate and it grows much more slowly in the absence of pantothenate than in its presence. Moreover, it synthesizes pantothenate after a considerable lag in Burkholder's medium; in Hutner's medium the lag is much shorter. Burkholder's medium is a much better diagnostic medium than Hutner's since growth occurs more rapidly on it than on Hutner's when pantothenate is supplied, but when pantothenate is not added, growth occurs less rapidly on Burkholder's than on Hutner's. That is, Burkholder's is a better medium when pantothenate is added but a poorer medium without added pantothenate.

THE "VITAMIN" PEDIGREE

Pedigree I (Table II) describes the melibiose-fermenting capacity of the various cultures which were subsequently investigated in some detail for their "vitamin-synthesizing" activity on Burkholder's medium according to his technique. The original diploid culture of S. *cerevisiae* (No. 1) was incapable of fermenting melibiose, and its offspring, cultures Nos. 3, 4, 5, and 6, were similarly incapable. In this pedigree numbers are used to indicate the different cultures, and each group of four in a single column includes the cultures produced by the four spores of a single ascus. Thus cultures 3, 4, 5, and 6 were originally from one diploid ascus of S. *cerevisiae.* Culture No. 2 (S. *carlsbergensis*) fermented melibiose, as did all of its haploid progeny. Culture No. 7 was the only survivor of a single ascus, and a mating between it and No. 4 produced a hybrid supposedly heterozygous for the ability to ferment melibiose.

This pedigree involves a non-Mendelian phenomenon which will be considered in greater detail in a later paper (Lindegren & Lindegren, '47). The pedigree is merely presented here for subsequent reference in tracing the descent of the different cultures.

HYBRIDS BETWEEN YEASTS SUPPOSEDLY DIFFERENT IN VITAMIN-SYNTHESIZING ABILITY

The members of this pedigree were characterized (Lindegren and Lindegren, '45) as "vitamin-synthesizers" and "vitamin-nonsynthesizers." The distinction between ability and inability to synthesize was made by Burkholder's method with his medium. This procedure defines readings taken at 72 hours as diagnostic. We have since discovered that this method does not give conclusive results, for if growth were allowed to continue, the supposedly nonsynthesizing yeasts will eventually begin to grow and will finally, in most cases, attain a level equal to that of the so-called synthesizers. However, at the end of 72 hours a 10- or 20-fold difference in growth often exists as indicated by reading the turbidity with a Klett Photoelectric Colorimeter. Haploids of S. *carlsbergensis* were characterized

TABLE I
FORMULAS OF DIFFERENT SYNTHETIC MEDIA

	Burkholder's	Hutner's	Beadle & Tatum's	Wickerham's
	Grams per liter			
Dextrose	20.0	20.0		10.0
Sucrose			20.0	
Recrystallized asparagine	2.0			1.0*
K_2HPO_4				0.125
KH_2PO_4	1.5	1.0	1.0	0.875
$MgSO_4.7H_2O$	0.5	0.2	0.5	0.5
$CaCl_2.2H_2O$	0.33	0.037	0.1	0.1
$(NH_4)_2SO_4$	2.0			1.0*
KI	0.0001			0.0001
NaCl			0.1	0.1
Sodium citrate		0.8		
Citric acid		0.2		
$(NH_4)_2HPO_4$		0.8		
Ammonium tartrate			5.0	
$(NH_4)NO_3$			1.0	
	Parts per million			
Boron	0.01		0.01	0.01
Manganese	0.01	0.5	0.02	
Zinc	0.07	4.0	2.00	0.07
Copper	0.01	0.1	0.10	0.01
Molybdenum	0.01		0.02	
Iron	0.05	4.0	0.20	0.05
	Micrograms per liter			
Thiamin	200	200		400
Pyridoxine	200	200		400
Nicotinic acid	200	200		400
Pantothenate	200	200		400
Biotin	2	2	5.0	2
Inositol	10,000	10,000		2,000
Riboflavin				200
p-amino-benzoic acid				200
	pH 5.0 with NaOH	pH 6.0 with citric acid		pH 5.3

* Either asparagine or $(NH_4)_2SO_4$ was used.

as biotin +, pantothenate +, and pyridoxine —; haploids of S. *cerevisiae* were characterized as biotin —, pantothenate —, and pyridoxine +, according to Burkholder's technique.

Table III shows the Klett readings obtained after 72 hours by Burkholder's technique of these different cultures. It appears that culture No. 4 is a non-synthesizer of pantothenate, while No. 7 is a synthesizer. A hybrid between No. 4

TABLE II

PEDIGREE OF A HYBRID BETWEEN *S. CEREVISIAE* (LK) AND *S. CARLSBERGENSIS* (MRAK, 126)

1		*S. cerevisiae* me diploid (Lk)	2		*S. carlsbergensis* Me diploid (Mrak, 126)		
3	a	me	7	α	Me	74	Me
4	a	me	76		Me	80	Me
5	α	me	77		Me	81	Me
6	α	me	78		Me	82	Me
			79		Me	83	Me

4 × 7 (a me × α Me)

10 diploid

20	α	Me	12	Me	16	Me	35	Me	90	Me	94	Me	110	Me
21	α	Me	13	Me	17	Me	36	Me	91	me	95	Me	111	me
22	a	Me	14	Me	18	Me	37	me	92	Me	96	Me	112	Me
23	a	Me	15	Me	19	Me	38	Me	93	Me				

4 × 20 (a me × α Me)

25	Me	29	Me	33	Me	39	me
26	Me	30	me	34	me	40	
27	me	31	Me	35	Me	41	Me
28	me	32		36	me	42	me

5 × 23 (α me × a Me)

45	Me	49	Me	53	me	57	Me	61		65		69	me	86	Me
46		50	me	54	me	58	me	62		66	me	70	Me	87	Me
47	me	51		55	Me	59	Me	63	me	67		71	Me	88	me
48	me	52		56		60	me	64	Me	68	Me	72	me	89	me

20 ×23 (α Me × a Me)

124	Me	128	Me	136	Me	140	Me	144	Me	150	
125	me	129	Me	137	Me	141	me	145	Me	151	Me
126	me	130	Me	138	Me	142	Me	146	Me	152	Me
127		131	Me	139	Me	143	Me			153	Me

4 × 49 (a me × α Me)		4 × 55 (a me × α Me)		
104	Me	117	me	121
105	me	118	Me	122
106	me	119		123
		120	Me	

5 × 72 (α me × a me)				7 × 72 (α Me × a me)	
98	me	101	me	Me	me
99	me	102	me	Me	Me
100	me	103	me	Me	Me
				Me	

and No. 7 (culture No. 10) appears capable of synthesizing pantothenate, but the four haploid progeny, Nos. 20, 21, 22, 23, do not reveal any segregation for this character according to this specific criterion. (The pantothenate character does segregate according to Mendelian ratios in other pedigrees which do normally segregate regularly.) It was subsequently discovered that none of these cultures was a nonsynthesizer and that the data appearing in Table III do not give an adequate picture of the synthetic ability of the organisms. The inadequacy of this characterization will be dealt with in detail below.

VITAMIN-SYNTHESIZING ABILITY OF SUPPOSEDLY DEFICIENT YEASTS IN A NATURAL MEDIUM

Dr. F. W. Tanner, Jr. (unpublished) grew various members of pedigree I (Table II) in a natural medium containing molasses and corn-steep liquor for 72 hours. When the yeast and the medium were assayed it was found that under these conditions all the cultures synthesized similar amounts of the different vitamins. Apparently the cultures were not clearly differentiated by ability and inability to synthesize the vitamins, but were all capable of synthesis under favorable conditions.

CONTINUOUS OBSERVATION OF GROWTH

Our present technique, an example of which has been presented in fig. 1, requires a much longer observation period. Many of the same cultures were reinvestigated and were sometimes observed for as long as a month, readings being made over the entire period. This is quite different from the standard practice of discontinuing the experiment after 72 hours. If the period of observation was extended for a longer time, haploid cultures of S. *cerevisiae* (as well as of S. *carlsbergensis*) were found to be able to grow on Burkholder's medium in the absence of pantothenate. This was equally true of all the progeny of the hybrid, many of which had previously been described as "nonsynthesizers." In some cases there was a delay of more than 600 hours before growth began. We grew the cultures in 6 x ¾-inch Kimble tubes and determined the amount of growth by measuring turbidity on the Klett Photoelectric Colorimeter adapted to take the larger tubes. This made it possible to make readings over the entire period without discarding them.

THE INADEQUACY OF THE CRITERION, EQUAL INCREMENTS OF GROWTH FOR EQUAL INCREMENTS OF VITAMIN

Culture No. 7 was planted in a series of media containing different concentrations of pantothenate, and the data produced a family of parallel curves (fig. 3 in following paper). If the experiment were terminated at an arbitrary time (standard practice in nearly all assay experiments), curves could be obtained in which equal increments of vitamin appear to produce equal increments of growth. The curves in fig. 2 were obtained by readings taken at various times. At 50 hours there appears to be a straight-line relation between the amount of added vitamin and

TABLE III

THE AMOUNT OF GROWTH AFTER 72 HOURS OF CULTURES FROM PEDIGREE 1 ON BURKHOLDER'S MEDIUM DEFICIENT IN THE INDICATED VITAMINS

Culture No.	Photometer Reading			Culture No.	Photometer Reading		
	—Py.	*—Pa.*	*—Bi.*		*—Py.*	*—Pa.*	*—Bi.*
1 (diploid)	350	220	11	*4*	350	15	12
2 (diploid)	24	280	280	*5*	310	20	8
				7	22	200	125
4 x *7*							
10 (diploid)	315	240	135				
20	274	45	50	*12*	355	293	170
21	312	202	55	*13*	80	65	160
22	290	200	59	*14*	350	220	75
23	300	210	110	*15*	25	15	7
90	318	212	140				
91	100	210	140				
92	300	235	134				
93	345	235	110				
4 x *20*							
25				*33*	340	12	10
26	310	25	145	*34*			
27	325	28	145	*35*	410	140	100
28				*36*	355	9	9
5 x *23*							
57	305	16	33	*69*	340	237	275
58	37	94	14	*70*	93	15	
59	38	100	10	*71*	358	16	230
60	347	16	20	*72*			
86	400	315	15	*154*	355	200	120
87	380	150	150	*155*	12	165	30
88	400	300	10	*156*	425	140	300
89	460	50	200	*157*	3	20	365
20 x *23*							
136	318	256	160				
137	360	222	193				
138	340	240	200				
139	320		170				

the amount of growth over a considerable portion of the curve. However, this culture was observed continuously, and eventually nearly as much growth was attained in the medium without the added vitamin as in the one containing relatively large amounts of added pantothenate (fig. 3 in following paper).

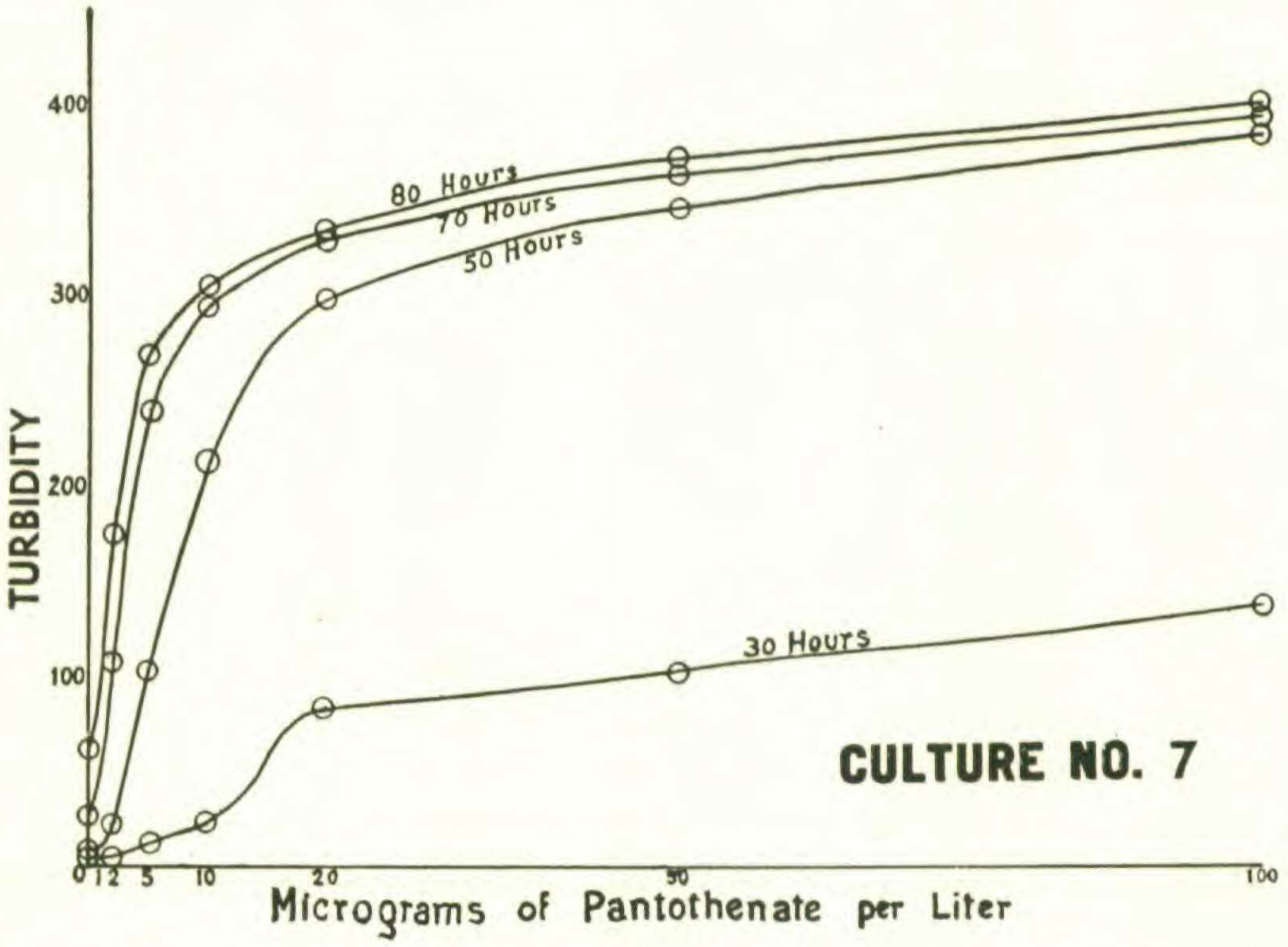

Fig. 2. The relationship between increments of pantothenate in Burkholder's medium and the amount of growth of culture No. 7 at different time intervals. In some phases of the curves a straight-line relationship exists between the amount of growth and the amount of added pantothenate but in all the culture tubes the growth eventually rose to approximately the same level.

THE EFFECT OF MULTIPLE DEFICIENCIES OF THE B VITAMINS

A haploid culture of *Saccharomyces cerevisiae* which grew well on an agar containing Hutner's synthetic medium with the vitamins, biotin, inositol, thiamin, and pantothenate, but which was unable to grow on the same agar medium lacking all these vitamins, was tested on agar containing various combinations of vitamins. Pour plates following Lederberg and Tatum's ('46) technique were made. In this technique a layer of sterile agar is poured in the bottom of the petri dish, then a layer of agar seeded with 500 cells poured on top, and a third layer of sterile agar on top of the second. This technique prevents colonies at the top and bottom of the agar from growing diffusely over or under the agar. A penicillin assay cup placed in the center of each plate was filled with a solution containing the combinations of vitamins.

Colonies were counted and a number of methods of scoring were tested, but simple + and — scoring is probably the most informative. The following tabulation gives the results:

VITAMINS PRESENT	VITAMINS ABSENT	SCORE
B I Th Pa		+ + + +
I Th Pa	B	+ + +
B Th Pa	I	+ + + + +
B I Pa	Th	+ +
B I Th	Pa	+ + +
Th Pa	B I	+ +
I Pa	B Th	+ +
I Th	B Pa	+ + +
B Pa	I Th	+
B Th	I Pa	—
B I	Th Pa	+
Pa	B I Th	+
Th	B I Pa	+
I	B Th Pa	+ +
B	I Th Pa	—
	B I Th Pa	—

The addition of inositol is apparently not very helpful in this concentration (10,000 γ per liter) when biotin, thiamin, and pantothenate were also present, for there was more growth when it was absent than when it was present. Actually there were fewer colonies when it was absent, but those which grew did much better. However, the culture supplied pantothenate alone grew less well than that supplied both inositol and pantothenate. There were probably considerable amounts of biotin in the agar, for removal of biotin did not usually reduce growth greatly. Removal of biotin and inositol simultaneously was serious. When either inositol or pantothenate were removed singly no serious effect occurred, but when both were removed together there was considerably less growth. The cells could synthesize both inositol and pantothenate easily when only one was absent but lacking both they synthesized poorly. These facts indicate that inability to grow in the absence of vitamins may involve simply lack of capacity to grow, or begin to grow, under the prescribed conditions rather than inability to synthesize the absent vitamins under all conditions (Williams, '41).

THE RELATION BETWEEN VIABILITY OF CELLS AND INABILITY TO GROW ON DEFICIENT MEDIUM

The ability or inability to grow in a deficient medium may merely involve inviability in the new medium rather than absolute inability to synthesize the vitamin in question. If the cells are inviable in the deficient medium they will be unable to begin synthesis of the required vitamin. The fact that most yeasts begin to synthesize when they are permitted to stand a sufficiently long time suggests that continued examination of cultures is necessary. This points up an important difference between *Neurospora* and yeasts. A conidium of *Neurospora* can put out a germ-tube and begin growth in distilled water, but if it is to continue growth it must be immediately supplied with the substances which it requires. It is relatively difficult to establish a *dormant mycelium* capable of awaiting future developments. Probably one of the most important advantages of a single-celled form over a filamentous fungus is that the single-celled form can go through one or two divisions and settle down to dormancy while a filamentous organism with specialized spores must continue to grow once the filament is produced or it will perish. This may give the appearance of absolute deficiencies to many mutants which die shortly after producing a filament in a medium lacking a specific vitamin.

These results have been supported by further work on three other mutants differentiated by ability to synthesize paraminobenzoic acid, pyridoxine, and uracil. So-called nonsynthesizers of pyridoxine eventually grew in a medium containing no added pyridoxine. Diploids heterozygous for a gene controlling synthesis of paraminobenzoic acid produced two classes of offspring, one which grew rapidly and one which grew slowly in the absence of paraminobenzoic acid. Although these two classes were clearly differentiated, the weak synthesizers were always able to grow eventually. An even more important bit of confirmatory evidence was obtained from the stock incapable of synthesizing uracil. The uracil-deficient cultures responded quickly to large amounts of uracil added to Burkholder's medium, but in the medium without added uracil they died. Transfer from uracil-deficient medium to a complete medium a few days after inoculation proved that the cells had died.

CONCLUSIONS

(1) Our results indicate that all so-called "nonsynthesizing" yeasts which remain alive when incubated in a deficient medium will eventually grow and synthesize vitamins in that medium.

(2) We have decided that Burkholder's conclusion that certain yeasts are "nonsynthesizers" requires qualification because synthesis might have been discovered if observation were made over a longer period.

(3) We suggest that many of Beadle and Tatum's "vitaminless" mutants may appear to be "nonsynthesizers" because they die in the deficient medium. "Reversions" of *Neurospora* mutants to wild-type, i. e., slow growth of "vitaminless" mutants in the deficient medium have been reported by Bonner, Tatum, and Beadle ('43) and other workers in this field, but they are usually regarded as exceptional cases rather than the standard expected behavior. The above results suggest the possibility that an improvement of the medium, without addition of the vitamin for which the stock is deficient, might lead to a higher frequency of reversions.